-DATA EVALUATION RECORD
AQUATIC INVERTEBRATE ACUTE TOXICITY TEST, FRESHWATER DAPHNIDS
GUIDELINE OPPTS 850.1010

1. **CHEMICAL:** Cetylpyridinium chloride (CPC) **PC Code No.:** 069160

2. **TEST MATERIAL:** Cetylpyridinium chloride (94.8%) **Purity:** 99.8%

3. **CITATION**

Authors: B Knight and CM Murphy
Title: CPC Determination of Acute Toxicity (EC_{50}) to Daphnia (48 h, Static)
Study Completion Date: March 3, 2005
Report Date: March 23, 2006
Laboratory: Inveresk, Tranent, EH33 2 NE, Scotland
Sponsor: Rutherford Chemicals LLC
Laboratory Report ID: 23187
MRID No.: 468162-04

4. **REVIEWED BY:**

Signature: **Richard C. Petrie, Agronomist – Team 3 Leader** **Date:** 1/23/08
RASSB/AD/OPP/OPPTS

5. **APPROVED BY:**

Signature: **Norm Cook, Chief** **Date:** 1/23/08
RASSB/AD/OPP/OPPTS

6. **STUDY PARAMETERS**

Scientific Name of Test Organism: *Daphnia magna*
Age of Test Organism: <24 hours
Definitive Test Duration: 48 hours
Study Method: Static
Type of Concentrations: Nominal

7. **CONCLUSIONS**

D. magna immobility percentages increased as CPC concentrations increased for both the range finding and definitive tests. In the range finding test, there was 20% immobility at the 0.1 µg/L concentration and 100% immobility in the 1, 10, and 100 µg/L concentrations. Since there were variable toxic effects observed in the range finding tests, an extended number of test concentrations were used in the definitive test. There was 60% immobility at 10 µg/L for the 24 hour definitive test and 75% immobility for the 48 hour definitive test. In the 32 µg/L and 100 µg/L concentrations of the definitive test, 100% immobility was observed for both the 24 and 48 hour windows.

Verified Results Synopsis:

48-hour EC_{50}: 7.36 µg/L (95% C.I. 3.2, 10.0 µg/L)
NOEC: 3.2 µg/L at 24 and 48 hours by observation
Binomial Test using TOXANAL

8. ADEQUACY OF THE STUDY

A. Classification: CORE

B. Rationale:

C. Repairability:

9. GUIDELINE DEVIATIONS:

The following guideline deviations were based on EPA OPPTS Guideline 850.1010:

- Acclimation period was not mentioned in the study
- Size of neonates was not provided
- Pretest mortality was not provided
- Health of *D. magna* prior to study commencement was not discussed
- Biomass loading rate was not discussed
- Use of solvents was not discussed
- Assignment methods were not discussed

10. SUBMISSION PURPOSE: Registration

11. MATERIALS AND METHODS

A. Test Organisms

Guideline Criteria	Reported Information
Species $ *Daphnia magna*	$ *Daphnia magna* (water flea)
Life Stage $ Daphnids: 1st instar (<24 h)	$ All neonates were <24 hours old at test initiation
All organisms from same source?	$ Bred within laboratory by acyclical parthenogenesis
Organisms approximately same size and age?	$ All neonates were <24 hours old at test initiation $ No data were provided on size
Signs of disease or injury?	$ No data were provided

Guideline Criteria	Reported Information
Acclimation Period Minimum 7 days	$ Acclimation period was not mentioned in the study
If treated for disease, was there no sign of the disease remaining during the 48 hours prior to testing?	$ No data were provided
Feeding No feeding during the study.	$ Fed *Chlorella vulgaris* (Strain 211/12, CCAP, Ambleside, Cumbria) prior to study initiation $ No feeding was reported during the test
Pretest Mortality No more than 3% mortality 48 hours prior to testing.	$ No mention of pretest mortality

B. Test System

Guideline Criteria	Reported Information
Source of dilution water $ Soft reconstituted water or water from a natural source, **not** dechlorinated tap water.	$ Diluted in Elendt M4 medium; 50 mL of stock solution of macronutrients (composition below), 0.1 mL vitamin stock (composition below), 0.294 mg/mL $CaCl_2 \cdot 2H_2O$, 0.123 mg/mL $MgSO_4 \cdot 7H_2O$, 0.0058 mg/L KCl, 0.0648 mg/L $NaHCO_3$, 0.001 mg/L $Na_2SiO_3 \cdot 9H_2O$, 0.00027 mg/L $NaNO_3$, 0.00014 mg/L KH_2PO_4, and 0.00018 K_2HPO_4. >2 hours aerated, pH 8.10 and conductivity 551 µS (range finding) and pH 7.27 and conductivity 486 µS (definitive test) $ Macronutrients stock solution prepared in deionized water (mg/L): 57.19 H_3BO_3, 7.21 $MnCl_2 \cdot 4H_2O$, 6.12 LiCl, 1.42 RbCl, 3.04 $SrCl_2 \cdot 6 H_2O$, 0.32 NaBr, 1.26 $Na_2MoO_4 \cdot 2H_2O$, 0.335 $CuCl_2 \cdot 2H_2O$, 0.26 $ZnCl_2$, 0.20 $CoCl_2 \cdot 6H_2O$, 0.065 Kl, 0.0438 Na_2SeO_3, 0.0115 NH_4VO_3, 10 $Na_2EDTA \cdot 2H_2O$, and 40 $FeSO_4 \cdot 2H_2O$) The 10 $Na_2EDTA \cdot 2H_2O$, and 40 $FeSO_4 \cdot 2H_2O$ were autoclaved prior to combination to the Elendt M4 medium. Vitamin stock solution (mg/L) 750 Thiamine hydrochloride, 10 Cyanocobalamine, and 7.5 Biotin. $ Ultrasonicated for *ca* 2-3 minutes to ensure dissolution
Does water support test animals without observable signs of stress?	$ No data were available
Photoperiod $ 16-hr light and 8-hr dark with 15- to 30-minute	$ 16-hours of light and 8 hours of dark using artificial daylight fluorescent tubes

Guideline Criteria	Reported Information
transition period.	
Test Aquaria $ Material: Glass or stainless steel. $ Size: 250 ml (daphnids and midges) or 3.9 L (1 gal). $ Fill volume: 200 ml (daphnids and midges) or 2-3 L.	$ Glass vessels with 200 mL capacity were used with perspex lids to prevent dust contamination and evaporation loss
Type of Dilution System $ Must provide reproducible supply of toxicant.	$ Test solutions were prepared by parallel dilutions of separate stock solutions of CPC (32 and 10 mg/L)
Water Temperature $ Daphnia: 20EC $ Amphipods and mayflies: 17EC $ Midges and mayflies: 22EC $ Stoneflies: 12EC	$ Test vessels were maintained within the range of 18.9-21.5°C $ Measured using a YSI 550 A (Yellow Springs Instrument) dissolved oxygen meter with a temperature probe
Dissolved Oxygen $ Static: ∃ 60% during 1st 48 h and ∃ 40% during 2nd 48 h $ Flow-through: ∃ 60%.	$ Kept within range of 71.8-96.5% of air saturation value $ Measured using a YSI 550 A (Yellow Springs Instrument) dissolved oxygen meter with a temperature probe
pH $ Prefer 7.2 to 7.6.	$ pH range 7.68-8.22 $ Measured with a Jenway 370 pH meter
Total Hardness $ Prefer 40 to 48 mg/L as $CaCO_3$.	$ Determined in a sample of the Elendt M4 medium used to prepare the test solutions in the definitive test as 220 mg/L $CaCO_3$
Flow Rate $ Consistent flow rate of 5-10 vol/24 hours $ Meter systems calibrated before study and checked twice daily during test period.	$ Static test substance delivery system
Biomass Loading Rate $ Static: # 0.8 g/L at # 17EC, # 0.5 g/L at > 17EC $ Flow-through: # 1 g/L/day.	$ No data were provided
Solvents $ Not to exceed 0.5 ml/L for static tests or 0.1 ml/L for flow-through tests.	$ No data were provided

C. Test Design

Guideline Criteria	Reported Information
Range Finding Test	*Initial range finding test:*

Guideline Criteria	Reported Information
$ If LC_{50} >100 mg/L, then no definitive test is required.	$ Initial range finding test conducted at nominal concentrations of CPC of 0, 0.1, 1, 10, or 100 mg/L $ 100% immobilization was observed after 24 hours for all treated groups; no control immobilizations *Repeat range finding test:* $ Repeat range finding test conducted over a 48 hour period at nominal concentrations of CPC of 0, 0.1, 1, 10, or 100 µg/L $ Test solutions were prepared by serial dilution of a 10 mg/L nominal CPC stock solution with Elendt M4 medium. $ Duplicate vessels were prepared at each test concentration, each with 100 ml of test solution $ 5 *D. magna* were added to each vessel within 30 minutes of preparation $ After 24 hours of exposure, 100% immobilization was seen at 1,10, and 100 µg/L $ At 48 hours of exposure, 20% of the *D. magna* were immobile at 0.1 µg/L $ No control species were immobile
Nominal Concentrations of Definitive Test $ Control & 5 treatment levels $ A geometric series with each concentration being at least 60% of the next higher one.	$ The definitive test was conducted at nominal concentrations of CPC of 0, 0.1, 0.32, 1, 3.2, 10, 32 or 100 µg/L
Number of Test Organisms $ Minimum 20/level may be divided among containers.	$ *Range Finding Test:* 25 organisms for each of 2 replicates (4 groups of 5 animals each, plus control group) for both 24 and 48 hour tests $ *Definitive Test:* 40 organisms for each of 4 replicates (7 groups of 5 animals each, plus control group) for both 24 and 48 hour tests
Test organisms randomly or impartially assigned to test vessels?	$ No mention of assignment methods
Water Parameter Measurements $ Temperature: Measured continuously or, if water baths are used, every 6 h, may not vary > 1EC. $ DO and pH: Measured at beginning of test and ever 48 h in the high, medium, and low doses and in the control.	$ Temperature, pH, conductivity, and dissolved oxygen concentrations were measured at 0, 24, and 48 hours in one replicate vessel at each test concentration during the definitive test
Chemical Analysis $ Needed if solutions were aerated, if chemical was	$ Test vessels were not aerated during the test $ Chemical analysis was not conducted on the prepared

Guideline Criteria	Reported Information
volatile, insoluble, or known to absorb, if precipitate formed, if containers were not steel or glass, or if flow-through system was used	test solutions since the test concentrations were below the reliable limit of detection of the analytical method (0.27 mg/L) $ However, stock solutions of CPC (32 and 10 mg/L) used to prepare the test solutions were subjected to chemical analyses at 0 and 48 hours $ Duplicate aliquots (*ca* 20 ml) were removed from the prepared stock solutions (32 and 10 mg/L) and control media for chemical analyses at 0 and 48 hours $ Test samples were analyzed according to the procedure established and validated under Inveresk Protocol No. 343191

12. REPORTED RESULTS

Guideline Criteria	Reported Information
Quality assurance and GLP compliance statements were included in the report?	$ Yes (p. 3,4, and 25)
Control Mortality X Static: #10% X Flow-through: #5%	X Initial range finding: 0% X Repeat range finding: 0% X Definitive test: 0% (for 24 and 48 hours)
Percent Recovery of Chemical	X No data were available
Raw data included?	X Yes (Appendix p. 17-24)

Dose Response

Immobility (Repeat Range Finding)

Nominal Test Concentration (µg/L)	Mean Measured Test Concentration (µg/L)	Number of Organisms	Cumulative Number Dead		
			Hour of Study		
			2	24	48
Control	Negative Control	10	N/A	0	0

Nominal Test Concentration (µg/L)	Mean Measured Test Concentration (µg/L)	Number of Organisms	Cumulative Number Dead		
			Hour of Study		
			2	24	48
0.1	N/A	10	N/A	0	2
1	N/A	10	N/A	10	10
10	N/A	10	N/A	10	10
100	N/A	10	N/A	10	10

Data from Table 1 on p. 17 in study
N/A= No data were available, due to doses were below detection limits

Immobility (Definitive)

Nominal Test Concentration (µg/L)	Mean Measured Test Concentration (µg/L)[a]	Number of Organisms	Cumulative Number Dead		
			Hour of Study		
			2	24	48
Control	ND	20	N/A	0	0
0.1	N/A	20	N/A	0	0
0.32	N/A	20	N/A	0	0
1.0	N/A	20	N/A	0	0
3.2	N/A	20	N/A	0	0
10	10.3	20	N/A	12	15
32	30.6	20	N/A	20	20
100	N/A	20	N/A	20	20

Data from Table 3 on p. 19 in study
[a]mean measured test concentration is the overall mean measured concentration from the 0 and 48 hour measurements
N/A= No data were available, due to doses were below detection limits

Statistical Results

Statistical Method: Authors used probit transformation to compare observed immobilization data at each time point with the nominal concentration (Finney 1971, 1978). A Pearson Chi-square test on the sum of squares for each data point indicated low heterogeneity in the data. The probit transformed data were then subjected to a regression procedure against logarithmically transformed concentrations when appropriate. The Davidon-Fletcher-Powell maximum likelihood algorithm was also used to obtain parameter estimates. The EC_{50} value was estimated from the fitted model.

Results Synopsis:

24-hour EC_{50}: 9.65 µg/L (95% C.I. 8.93, 10.4 µg/L)
48-hour EC_{50}: 9.18 µg/L (95% C.I. 8.52, 9.90 µg/L)
NOEC: 3.2 µg/L at 24 and 48 hours

13. VERIFICATION OF STATISTICAL RESULTS:

Definitive (24 hour)

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 32              20              20              100             9.536742E-05
 10              20              12              60.00001        25.17223
 3.2             20              0               0               9.536742E-05
 1               20              0               0               9.536742E-05
 .32             20              0               0               9.536742E-05
 .1              20              0               0               9.536742E-05

THE BINOMIAL TEST SHOWS THAT 3.2 AND 32 CAN BE
USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT
CONFIDENCE LIMITS, BECAUSE THE ACTUAL CONFIDENCE LEVEL
ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.


AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 8.678638


WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE
PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE NOR THE
PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.
```

Note: The whole screenshot did not display correctly in TOXANAL (missing 100 µg/L group, but the analyses were run correctly.

24-hour EC_{50}: 8.68 µg/L (95% C.I. 3.2, 32 µg/L)
NOEC: Not available

Definitive (48 hour)

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 32              20              20              100             9.536742E-05
 10              20              15              75              2.069473
 3.2             20              0               0               9.536742E-05
 1               20              0               0               9.536742E-05
 .32             20              0               0               9.536742E-05
 .1              20              0               0               9.536742E-05

THE BINOMIAL TEST SHOWS THAT 3.2 AND 10 CAN BE
USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT
CONFIDENCE LIMITS, BECAUSE THE ACTUAL CONFIDENCE LEVEL
ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.


AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 7.360023


WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE
PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE NOR THE
PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.
```

Note: The whole screenshot did not display correctly in TOXANAL (missing 100 µg/L group), but the analyses were run correctly.

48-hour EC_{50}: 7.36 µg/L (95% C.I. 3.2, 10.0 µg/L)
NOEC: Not available

14. REVIEWER=S COMMENTS:

The study seemed adequate to determine the EC_{50} for *D. magna*. GLP and quality assurance statements were included in the study and the protocol deviations seemed minor and unlikely to influence the study results. The extended number of nominal concentrations in the definitive test was appropriate given the results of the range finding tests. Although no other health endpoints were discussed, the immobility data was clear and sufficient for the goal of the study. In verifying the statistical results, there was some discrepancy between the results in the study and the review and may be due to difference in calculation techniques. These differences are not extremely large and considered acceptable. Mortality and health of the animals prior to testing could be considered major discrepancies; however, there was not a large number of deaths noted in the control groups and not thought to interfere with the results of the test.

References

Finney, D.J. (1971) Probit Analysis, 3rd Edition. London: Charles Griffin and Company.

Finney, D.J. (1978), Statistical Methods in Biological Assay, 3rd Edition. London: Charles Griffin and Company.